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Analysis of the roles of E6 binding to E6TP1 and nuclear localization in the human papillomavirus type 31 life cycle

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Abstract

The E6 oncoproteins of high-risk human papillomaviruses provide important functions not only for malignant transformation but also in the productive viral life cycle. E6 proteins have been shown to bind to a number of cellular factors, but only a limited number of analyses have investigated the effects of these interactions on the viral life cycle. In this study, we investigated the consequences of HPV 31 E6 binding to E6TP1, a putative Rap1 GAP protein. HPV 16 E6 has been shown to bind as well as induce the rapid turnover of E6TP1, and similar effects were observed with HPV 31 E6. Mutation of amino acid 128 in HPV 31 E6 was found to abrogate the ability to bind and degrade E6TP1 but did not alter binding to another α-helical domain protein, E6AP. When HPV 31 genomes containing mutations at amino acid 128 were transfected into human keratinocytes, the viral DNAs were not stably maintained as episomes indicating the importance of this residue for pathogenesis. Many E6 binding partners including E6TP1 are cytoplasmic proteins, but E6 has been also reported to be localized to the nucleus. We therefore investigated the importance of E6 localization to the nucleus in the viral life cycle. Using a fusion of E6 to Green Fluorescent Protein, we mapped one component of the nuclear localization sequences to residues 121 to 124 of HPV 31 E6. Mutation of these residues in the context of the HPV 31 genome abrogated the ability for episomes to be stably maintained and impaired the ability to extend the life span of cells. These studies identify two activities of HPV 31 E6 that are important for its function in the viral life cycle and for extension of cell life span.

Keywords: Viral life cycle; HPV; Nuclear localization; E6TP1

Introduction

Human papillomaviruses (HPVs) are small DNA viruses that contain double-stranded circular genomes of approximately 8000 base pairs in size. HPV infection is causally associated with the development of various hyperproliferative diseases of the epithelia (Howley and Lowy, 2001). In particular, infection by the high-risk HPVs types such as HPV 16, 18, 31, and 54 is the single most important risk factor for the development of the cervical cancers (Laimins, 1993; Lowy et al., 1994; zur Hausen, 2002; zur Hausen and de Villiers, 1994). This association with malignancy is dependent on the action of the two viral oncoproteins, E6 and E7. The E6 protein mediates the turnover of the tumor suppressor protein p53 (Huibregtse et al., 1991; Scheffner et al., 1990, 1993; Werness et al., 1990), while E7 binds and inactivates the retinoblastoma (pRb) family of proteins (Cheng et al., 1995; Dyson et al., 1989; Martin et al., 1998; Munger et al., 1989). In addition, several studies have identified p53- and pRb-independent activities of E6 and E7, which are also important for HPV-induced oncogenesis (Brehm et al., 1999; Kiyono et al., 1998; Klingelhutz et al., 1996; Liu et al., 1999).

The life cycle of HPV is closely linked to the differentiation program of the host keratinocyte (Howley and Lowy, 2001). HPVs infect keratinoctyes in the basal layer that become exposed following microtraumas to the epithelia. Following entry, viral genomes are established as episomes and replicate together with the host DNA through the action of cellular replication proteins working with the viral factors, E1 and E2 (Stubenrauch and Laimins, 1999). Following cell division, one of the infected daughter cells migrates away from the basal layer and undergoes differentiation. Upon initiating differentiation,

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normal keratinocytes exit the cell cycle while HPV-positive cells continue to cycle through the action of the E7 protein (Cheng et al., 1995; Flores et al., 1999; Halbert et al., 1992). In highly differentiated suprabasal cells, late viral functions are activated including the amplification of viral genomes and the expression of the late viral proteins (Cheng et al., 1995; Flores et al., 1999; Halbert et al., 1992; Hummel et al., 1992). The E6 protein plays an important role in the viral life cycle by contributing to the stable maintenance of viral episomes in undifferentiated cells (Thomas et al., 1999) and may mediate additional effects in differentiated suprabasal cells.

Recent studies have demonstrated that the binding of E6 to cellular proteins other than p53 is important for viral pathogenesis. The binding of HPV 31 E6 to PDZ proteins through a motif present at its C-terminus was shown to be important for mediating early viral functions in undifferentiated cells (Lee and Laimins, 2004). A number of additional E6-interacting proteins have been identified that contain a seven amino acid motif which forms part of an α-helical structure (Be et al., 2001; Chen et al., 1998). One of these α-helical domain-containing proteins is E6AP, an E3 ubiquitin ligase necessary for E6-mediated degradation of p53 (Huibregtse et al., 1991; Scheffner et al., 1990, 1993; Werness et al., 1990). Other α-helical domain-containing proteins that have been shown to bind to E6 include E6BP (Chen et al., 1995), a calcium-binding protein of the CREC family, and E6TP1 (Gao et al., 1999), a putative Rap1 GTPase activating protein (GAP).

Studies have shown that the ability of HPV 16 E6 to immortalize human mammary epithelial cells correlated with its ability to bind and degrade E6TP1 (Gao et al., 1999; Gardiol et al., 1999). Furthermore, transgenic mice expressing an HPV 16 E6 mutant defective for binding to E6AP and E6BP exhibited a reduced oncogenic potential in vivo (Nguyen et al., 2002). In contrast, another study reported that mutants of E6 which exhibited reduced binding to E6AP and E6BP were still able to induce the immortalization of mammary epithelial cells (Liu et al., 1999). It is unclear if all high-risk types HPV E6 proteins bind these α-helical domain-containing proteins and what the physiological significance of these interactions is to HPV life cycle.

The high-risk E6 proteins are localized to both the cytoplasm and the nucleus. E6TP1, E6AP, and E6BP are all found in the cytoplasm, while the PDZ-domain-containing proteins are detected at cytoplasmic membranes. Additional binding partners of E6 have been identified that are nuclear in location. These include transcriptional regulators such as c-Myc (Gross-Mesilaty et al., 1998), ADA3 (Kumar et al., 2002), CBP/p300 (Patel et al., 1999), IRF-3 (Ronco et al., 1998), MCM7 (Kukimoto et al., 1998), and DNA repair proteins such as XRCC-1 (Iftner et al., 2002), and *O*(6)-MGDMT (Srivenugopal and Ali-Osman, 2002). This suggests that nuclear localization of E6 may be required for some function in the viral life cycle. While it has been reported that HPV 16 E6 contains a complex nuclear localization signal (NLS) consisting of three distinct domains (Tao et al., 2003), another study indicated that one of these motifs alone was sufficient for nuclear localization (Le Roux and Moroianu, 2003). In the present study, we have examined two different functions of HPV 31 E6 in the viral life cycle. We determined that HPV 31 E6-mediated degradation of E6TP1 is important for the stable maintenance of viral episomes and the extension of cell life span. In addition, we mapped one component of the nuclear localization signal of HPV 31 E6 and demonstrated that this sequence was important for E6's function in the viral life cycle.

Results

A threonine substitution at the isoleucine 128 of HPV 31 E6 inhibits the E6-mediated degradation of E6TP1

In order to begin to investigate the functions of E6 that are important for its role in the viral life cycle, we have utilized genetic analyses in the context of complete viral genomes. The first step in such studies is the identification of mutations in E6 that interfere with binding of cellular factors or other activities. With this goal in mind, we focused on HPV 31 E6 interactions with the α-helical domain protein, E6TP1, which is a putative Rap1 GAP protein. It was previously demonstrated that mutation of the isoleucine at amino acid 128 in HPV 16 E6 abrogated its ability to bind proteins that contain α-helical domains (Liu et al., 1999). This amino acid is conserved in HPV 31 E6, and we investigated the effects of mutating this residue on interactions with E6TP1 (Fig. 1). For these studies, we used transient transfection of myc-tagged E6TP1 expression vectors together with plasmids encoding either wild-type HPV 31 E6 or a mutant E6 encoding a threonine at position 128 instead of an isoleucine (I128T). At 48 h after transfection, total protein lysates were prepared and analyzed by Western blot analysis using an antibody to myc. As shown in Fig. 2A, two bands were detected by Western analysis using extracts from cells transfected with expression vectors for myc-tagged E6TP1 (quantified in Fig. 2B). The slowest migrating band corresponds

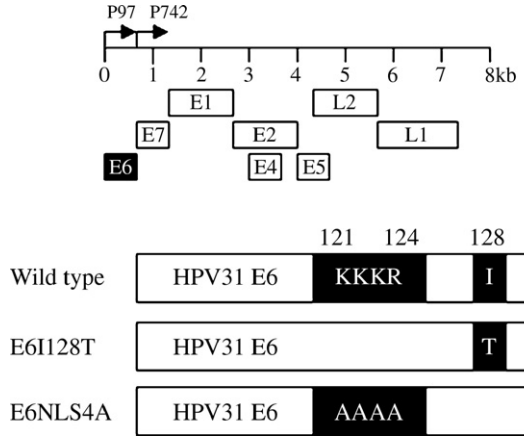

Fig. 1. Diagram of a linearized HPV 31 genome identifying the viral open reading frames and the two major viral promoters (P97 and P742). Schematic depicting the amino acid residues of HPV31 E6 important for binding to the E6TP1 and its nuclear accumulation. The two E6 mutant genomes were examined in this study; E6I128T contains a substitution of the 128th isoleucine with threonine while E6NLS4A contains a substitution of three lysines and arginine from amino acid 121 to 124 with four alanines.

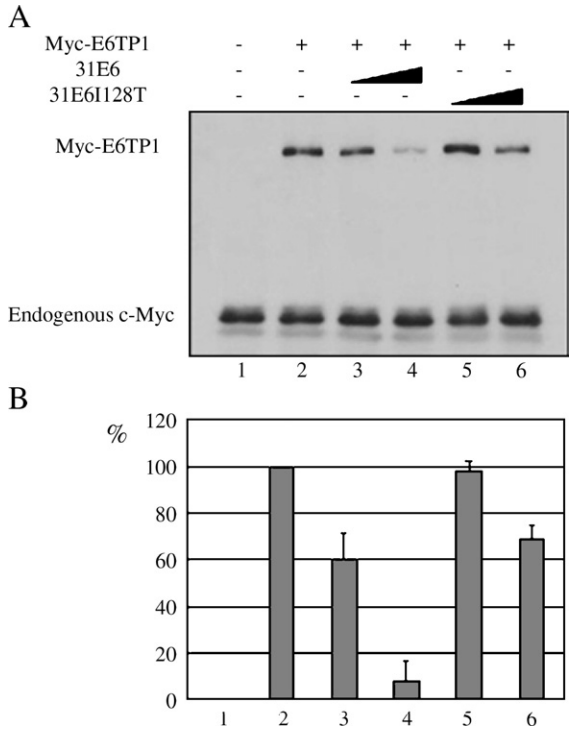

Fig. 2. (A) Western blot analysis of myc-tagged E6TP1 proteins in the presence of HPV 31 E6 wild-type or E6I128T proteins in C33A cells. Myc-tagged E6TP1 expression vectors were transfected either alone or together with increasing amount of HPV 31 E6 wild-type as well as HPV 31 E6I128T expression vectors into C33A cells. At 48 h after transfection, equivalent amounts of whole-cell extracts from each of the transfected cells were isolated and separated on an SDS-polyacrylamide gel and transferred to membrane. This membrane was probed with anti-myc antibody, and proteins were visualized by chemiluminescence. Lane 1: control; lane 2: 1 μg of myc-E6TP1 plasmid; lane 3: 1 μg of myc-E6TP1 plasmid+1 μg of HPV 31 E6; lane 4: 1 μg of myc-E6TP1+2 μg of HPV 31 E6 plasmids; lane 5: 1 μg of myc-E6-TP1+1 μg of HPV 31 E6 I128T plasmid; lane 6: 1 μg of myc-E6TP1+2 μg of HPV 31 E6 I128T plasmids. Total DNA was normalized to 3 μg per transfection. (B) Quantification of data in panel A.

to myc-tagged E6TP1, while the faster migrating band corresponds to the endogenous myc protein. In the untransfected control lane, only the faster migrating band was seen (lane 1). When an myc-tagged E6TP1 vector was cotransfected with an equal amount of HPV 31 E6 expression vector, we observed an approximate 40% reduction in levels of the tagged protein (lane 3). As the amount of cotransfected HPV 31 E6 expression plasmid was increased two-fold, we observed a 90% decrease in the levels of tagged E6TP1 (lane 4). When equal amounts of the tagged E6TP1 expression construct were cotransfected with HPV31 E6 plasmid in which amino acid 128 was altered to threonine, no decrease in the levels of E6TP1 was seen (lane 5). However, cotransfection of higher amounts of HPV 31 E6I128T plasmids with tagged E6TP1 expression vectors resulted in an approximate 30% reduction in levels of the tagged protein (lane 6). This suggests that mutation of amino acid 128 significantly impairs the ability of HPV31 E6 to degrade E6TP1.

For HPV 16 E6, mutation of amino acid 128 has been reported to block binding to E6AP and thus prevent its degradation (Liu et al., 1999). We investigated whether similar effects were seen with the HPV 31 E6 protein and used an HA-tagged E6AP expression vector that has been used previously for the HPV 16 studies (Gardiol et al., 1999). When HPV 31 E6 expression vectors were cotransfected with plasmids expressing HA-E6AP into cells, reduced levels of tagged E6AP were seen by Western analysis as compared to those detected in transfections with HA-E6AP vector alone (Fig. 3A). Interestingly, when we cotransfected the HPV 31 I128T E6 and HA-E6AP vectors, we observed a similar reduction in levels as seen in cotransfections with HPV 31 E6 (Fig. 3A). This experiment was repeated twice with similar results. We conclude that introduction of the I128T mutation into HPV 31 E6 does not alter its ability to degrade to E6AP. We next investigated the ability of HPV 31 E6 and E6I128T to bind E6AP. For this purpose, we used in-vitro translated HPV 31 E6 and E6I128T

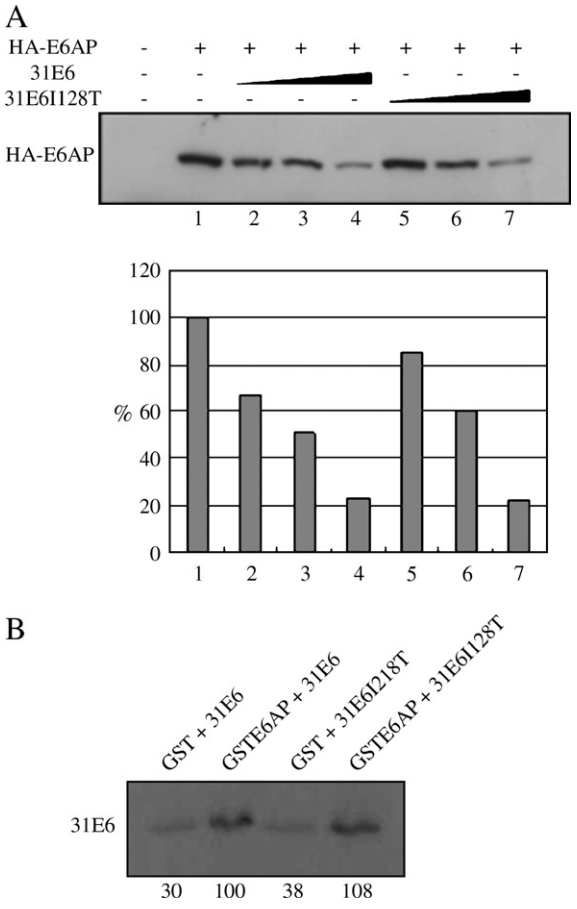

Fig. 3. (A) Western blot analysis of HA-tagged E6AP proteins in the presence of HPV 31 E6 wild-type or E6I128T proteins in C33A cells. HA-tagged E6AP expression vectors were transfected either alone or together with increasing amount of HPV 31 E6 wild-type as well as HPV 31 E6I128T expression vectors. At 48 h after transfection, equivalent amounts of whole-cell extracts were subjected to Western blot analysis using anti-HA antibody. A graph providing quantitation of the data is shown. (B) GST pull-down assay examining the interaction of HPV 31 E6 and E6I128T with E6AP. GST and GST-E6AP fusion proteins conjugated with the glutathione–agarose beads were mixed with in-vitro translated HPV 31 E6 and E6I128T proteins radiolabeled with [^{35}S] cysteine. Bound proteins were eluted and separated on an SDS-polyacrylamide gel for autoradiography. Quantitation of the data relative to E6TP1 binding to wild-type E6 (set to 100) is indicated below lanes.

proteins and GST and GST-E6AP fusion proteins in GST pull-down assays. In previous studies with HPV 16 E6, E6AP binding was shown to be dependent upon amino acid 128, however, in our study, no differences were detected in the binding of E6AP to wild-type or mutant HPV 31 E6 proteins (Fig. 3B). We conclude that, while HPV 31 E6 binds and induces the degradation of E6AP, it does not depend on amino acid 128 for this activity. Similarly, E6BP has also been shown to bind HPV 16 E6 and that alteration of amino acid 128 of E6 abrogates its ability to bind to E6BP (Liu et al., 1999). When we performed pull-down experiments with GST-HPV 31 E6 in the presence of cell extracts, no association with E6BP was observed (data not shown).

It was next important to demonstrate that the introduction of threonine in place of isoleucine did not change the half life of HPV 31 E6. We are not able to immunoprecipitate HPV 31 E6 proteins from cells using antibodies or to observe these proteins in Western analyses, and so we have relied on the use of tagged proteins. For these studies, we have used tagged HPV 31 E6 with Green Fluorescent Protein (GFP). Vectors expressing GFP-tagged HPV 31 E6 wild-type and GFP-tagged E6I128T mutant proteins were transfected into cells. At 48 h after transfection, cell lysates were examined for E6 levels in Western analyses using a GFP antibody. As seen in Fig. 4, no significant differences were seen in the levels of wild-type or E6I128T proteins. We performed similar determinations of half life using an HA-tagged HPV18 E6 vector and found that the 128 mutation had no effect on stability (data not shown). For technical reasons that are unclear, we were not able to express HA-tagged HPV 31 E6 proteins.

HPV 31 E6I128T mutant genomes do not extend cell life span and fail to stably maintain episomes

We next investigated the effects of introducing the I128T mutation in the context of the complete HPV 31 genome on the ability to extend the life span of transfected human keratinocytes. It has been shown that, when HPV 31 genomes are recircularized and transfected into keratinocytes, HPV-positive cells exhibit extended life spans and become immortal (Frattini et al., 1996). For the current analysis, we recircularized both wild-type and E6I128T mutant genomes and transfected them into normal human keratinocytes along with a neomycin drug resistance marker. Following drug selection, colonies were pooled and passaged. As shown in Table 1, cells transfected with HPV 31 E6I128T mutant genomes or drug resistance marker alone senesced after a few passages in culture, while cells containing wild-type HPV 31 could be passaged greater than 20 times. These experiments were repeated three times with independently transfected host keratinocytes derived from different donors and observed similar results. We conclude that mutation of amino acid 128 of HPV 31 E6 impairs the ability of the viral genome to extend the life spans of the transfected cells.

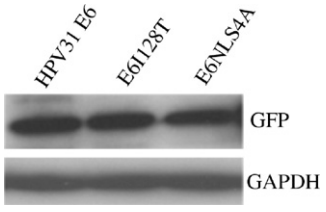

Fig. 4. Western blot analysis of cell lysates of 293-T cells transiently transfected with HPV 31 E6 GFP fusion plasmids: pSG5-GFP-WTE6, pSG5-GFP-WTE6I128T and pSG5-GFP-E6NLS4A. Whole-cell lysates were harvested at 48 h post-transfection and subjected to Western blot analysis using an anti-GFP antibody. Corresponding proteins are indicated: HPV31 wild-type E6, I128T mutant E6, or NLS mutant E6.

Table 1
Comparison of maximum passage numbers of the HFKs transfected with the neomycin resistance gene only or together with either HPV 31 wild-type or one of two mutant genomes (E6I128T and E6NLS4A) after a drug selection

Cell line	A	B	C
Neo control	3	2	1
HPV31	>20	>20	>20
E6I128T	3	2	2
E6NLS4A	5	4	4

Three different HFKs isolates used for the stable transfection were referred to as A, B, and C, respectively.

It was also important to examine the state of viral DNA in these transfected cells prior to senescence. Total DNA was isolated from keratinocytes transfected with wild-type and E6I128T mutant genomes at passage two following selection (greater than 17 cell doublings) and examined by Southern blot analysis for the state of viral DNA. As shown in Fig. 5A, only wild-type HPV 31 genomes were stably maintained as episomes. In cells transfected with E6I128T mutant genomes, the viral DNA was found to be integrated at very low copy numbers. Similar results were seen in three independent transfections (data not shown). We conclude that amino acid 128 of HPV 31 E6 is important for the stable maintenance of the HPV31 viral genomes.

As a control, we sought to confirm that the alteration of amino acid 128 did not result in a *cis* mutation that altered stability of transcripts encoding the replication proteins E1 and E2, which could indirectly lead to loss of replication ability. Previous studies demonstrated that transcripts encoding E1 and E2 initiate at the early promoter and include sequences in the E6 open reading frame (Hubert and Laimins, 2002). While genomes containing stop codons in E6 were shown to have little effect on transient replication, mutation of the E6 splice donor resulted in reduced expression of E1 leading to impaired replication (Hubert and Laimins, 2002; Thomas et al., 1999). We used this same assay to investigate if the E6I128T mutation exerts a *cis* effect that was responsible for reduced ability to stably replicate genomes. As shown in Fig. 5B, at 5 days after transfection into SCC-13 cells, the mutant genomes were able to replicate at levels comparable or even slightly higher than the wild-type genome. This indicates that the E6I128T mutation does not result in a *cis* defect in the replication ability of the HPV 31 genome. Overall, our studies indicate that the introduction of the I128T mutation into HPV 31 genomes significantly impairs the ability of viral DNA to be stably maintained as episomes and to extend the life span of cells.

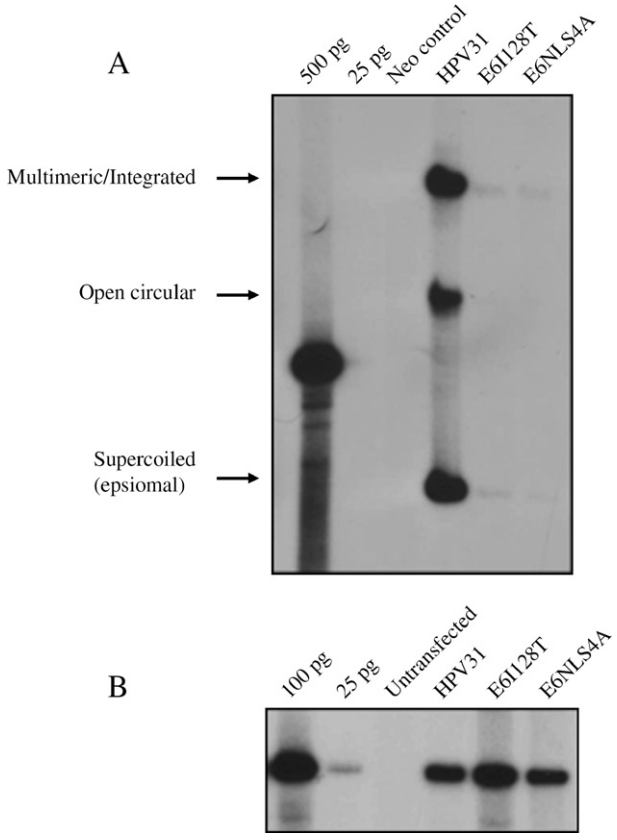

Fig. 5. (A) Southern blot analysis of HFKs stably transfected with HPV 31 wild-type, E6I128T, and E6NLS4A genomes following selection and expansion in monolayer cultures. First and second lanes contain 500 pg and 25 pg of linearized HPV 31 genome as copy number control, respectively. Next, four lanes contain 10 μg of uncut total DNA isolated from cells transfected with a neomycin gene only or together with HPV 31 wild-type and two mutant genomes. The multimeric as well as the episomal forms of the viral genomes are indicated at the left side of the autoradiograph. A broad band of low intensity is present in the lanes containing DNA isolated from cells transfected with mutant genomes. Viral copy numbers in the cells with integrated genomes are significantly reduced from that seen in cells containing wild-type genomes. (B) Southern blot analysis of SCC-13 cells transiently transfected with HPV 31 wild-type, E6I128T, and E6NLS4A genomes as well as an untransfected control. First and second lanes contain 100 pg and 25 pg of linearized HPV 31 genome as copy number control, respectively.

HPV 31 E6 contains a nuclear localization signal that is dependent upon amino acids 121–124

Previously we demonstrated that the binding of E6 to the PDZ domain proteins, which are localized at cytoplasmic membranes, provides an important activity in the HPV 31 life cycle (Lee and Laimins, 2004). In the present study, we observed that the interaction of HPV 31 E6 with the cytoplasmic protein E6TP1 also plays an important role. However, E6 has been reported to localize to both the cytoplasm as well as the nucleus (Grossman et al., 1989; Kanda et al., 1991; Sherman and Schlegel, 1996). We therefore wanted to investigate if the activities of E6 in the nucleus were necessary for some aspect of the viral life cycle. In studies examining HPV 16 E6, it was shown that amino acids 121–124 (KKQR) are important for its nuclear localization (Fig. 1B) though the complete signal may actually be a multicomponent element. Sequence comparison of HPV 31 and 16 E6 proteins identified a similar domain of positively charged amino acids in the HPV 31 E6 protein (121–124 KKKR). We investigated if these residues were important for HPV 31 E6 localization to the nucleus by constructing a fusion of E6 with Green Fluorescent Protein (GFP) coding sequences inserted in frame at the N-terminus (GFP-31E6). In addition, a mutant GFP-31E6 expression vector was constructed in which amino acids 121–124 were changed to four alanines (GFP-31E6NLS4A). Plasmids expressing GFP alone, GFP 31E6, and GFP 31E6NLS4A were transfected into cells, and the subcellular localization of the GFP-fused proteins was examined by fluorescence microscopy. As shown in Figs. 6A and B, expression of GFP by itself resulted in a uniform distribution throughout the cell, while the GFP 31E6 protein was found to localize largely to the nucleus with a minor population in the cytoplasm (Figs. 6C and D). This is in agreement with published data on the localization of HPV 16 and 18 E6 proteins (Kanda et al., 1991; Sherman and Schlegel, 1996). In cells transfected with the GFP 31E6NLS4A construct, a uniform distribution of signal was observed throughout the cell and this was markedly different from what was observed with the GFP 31E6 protein (Figs. 6E and F). Mutation of the KKKR motif HPV 31 E6 reduced the amount accumulated in the nucleus resulting in a fairly uniform distribution throughout the cell. We conclude that the residues 121–124 in HPV 31 E6 are important for nuclear localization, though additional sequences may also be required.

In order to investigate if mutation of amino acids 121–124 altered protein stability, we performed similar experiments to those already described for the I128T mutant of E6. In this analysis, expression vectors for GFP 31E6 and GFP 31E6NLS4A were transiently transfected into cells. At 48 h post-transfection, cell lysates were prepared and the levels of GFP-tagged E6 were examined by Western blot analysis using an antibody to GFP. As shown in Fig. 4, no significant differences in amount of E6 wild-type and mutant proteins were detected. This suggests that mutating the charged amino acids at amino acids 121–124 does not alter the stability of E6.

Amino acids 121–124 of HPV 31 E6 are important for extended life span and stable replication of the viral genomes

We next examined if alteration of HPV 31 E6 nuclear localization affected aspects of the viral life cycle. For these studies, we introduced the aa 121–124 mutation into E6 in the context of the complete HPV 31 genome. The recircularized wild-type and mutant genomes were then transfected into normal human keratinocytes along with a drug selectable marker as described in Materials and methods. Pooled drug-resistant cells were isolated and examined for the ability to be passaged for extended periods of time. Cells containing genomes with the 121–124 mutation senesced after several passages (Table 1). When we examined the state of viral DNA in the cells containing 121–124 mutant genomes at early passage, the genomes were found to be integrated at low copy numbers (Fig. 5A). These

experiments were repeated in three independently generated transfected cell lines, and similar results were observed. Finally, we confirmed that the 121–124 mutation in E6 does not exert a *cis* effect on E1/E2 gene expression by performing transient transfection assay as described previously. As shown in Fig. 5B, no effect of the mutation was observed in the short-term replication assay demonstrating that the 121–124 mutation does not have a *cis* effect on replication. Overall, our studies indicate that localization of E6 to the nucleus is important for several aspects of the HPV life cycle such as extension of cell life span and stable maintenance of viral episomes.

Discussion

In the present study, we investigated the importance of HPV 31 E6 induced degradation of the α-helical domain-containing protein E6TP1 on the viral life cycle. Previous studies suggested that high-risk E6 proteins may associate with the α-helical domain-containing proteins, such as E6TP1, E6BP and E6AP, through similar sequences (Be et al., 2001; Chen et al., 1998). Our studies indicate that differences exist between HPV 16 E6 and HPV 31 E6 in these properties, and this has allowed us to examine the contribution of HPV 31 E6 interactions with

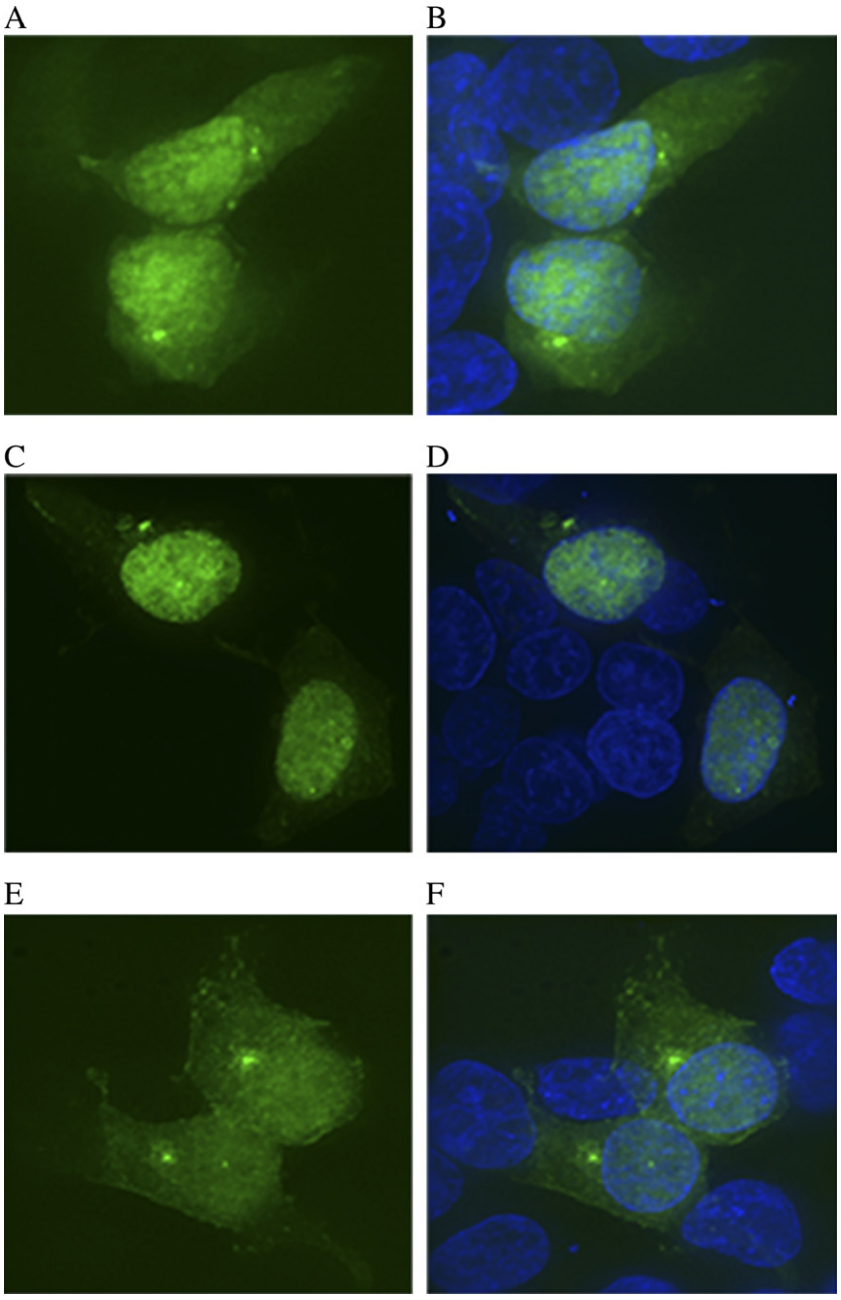

Fig. 6. Subcellular localization of GFP in pSG5-GFP (A, B), pSG5-GFP-WTE6 (C, D), and pSG5-GFP-E6NLS4A (E, F) in 293-T cells, 24 h post-transfection. Images were obtained using an inverted fluorescence microscope and deconvolved for higher image quality. Images A, C, and E are shown with GFP fluorescence only, and images B, D, and F show the merged GFP and DAPI fluorescence.

E6TP1, E6AP and E6BP independently. We observed that the binding and degradation of E6TP1 by HPV 31 E6 are required to extend the life span of human keratinocytes as well as for the long-term, stable replication of the viral genome. These two properties may be inter-related such that the enhanced proliferation of keratinocytes may be necessary to facilitate maintenance of episomes as senescing cells seem to quickly lose this ability. In addition, normal cells do not tolerate the long-term presence of extrachromosomal DNA and E6 proteins may target the corresponding surveillance mechanisms to allow for maintenance of HPV episomes. E6-mediated degradation of E6TP1 may play a direct or indirect role in overcoming these surveillance mechanisms to allow for the maintenance of HPV episomes.

Our studies also indicate that HPV 31 E6, like HPV 16 E6, binds to E6AP and mediates its auto-degradation. However, the interaction domain is not the same as that for E6TP1. While previous studies had suggested that degradation of E6TP1 occurred through E6AP (Gao et al., 2002), our studies indicate that E6AP binding alone is not sufficient for this activity and that other factors are probably involved. Whether E6AP plays any role in HPV 31 E6-mediated degradation of E6TP1 or contributes to E6's role in the viral life cycle is not clear at this time. A previous study by Park and Androphy demonstrated that degradation of p53 was necessary for maintenance of HPV 16/31 chimeric genomes while E6AP binding alone was not (Park and Androphy, 2002). A second α-helical domain-containing protein, E6BP, that was shown to be a binding partner of HPV 16 E6 was found not to bind to HPV 31 E6. Therefore, our studies suggest that all high-risk E6 proteins do not bind to the α-helical domain-containing proteins in a similar manner. Overall, our studies indicate that degradation of E6TP1 by E6 is important in the HPV 31 life cycle.

The E6TP1 protein is expressed in many cell types and contains a GAP domain that selectively interacts with Ras-related small G proteins called Raps. HPV 16 E6 has been shown to inhibit the GAP function of Rap1 and 2, resulting in enhanced GTP loading of Rap (Singh et al., 2003). Expression of Rap1 has also been shown to revert the transformed phenotype of viral K-ras-transformed NIH3T3 cells (Kitayama et al., 1989). Furthermore, Rap1 binds to Raf-1 as well as the catalytic subunit of phosphotidylinositol 3-kinase and so antagonizes Ras function (Frech et al., 1990; Hata et al., 1990; Herrmann et al., 1996). E6-mediated degradation of E6TP1 may lead to increased cell proliferation through inhibition of GAP activity of E6TP1 toward Rap proteins, and this is likely to be important for HPV pathogenesis. It will be interesting to see what effect blocking E6TP1 function in normal keratinocytes by siRNA treatment has on cell proliferation and whether this can complement E7 function in inducing cell immortalization. It is possible that E6TP1 and p53 degradation are two important properties mediated specifically by high-risk HPVs E6 protein to allow for viral replication.

Many of the factors that have been shown to bind E6 are localized to the cytoplasm and plasma membranes. However, our studies indicate that E6 also has activities in the nucleus that are important for viral pathogenesis. We identified a region of 4 amino acids (121–124) that is important for HPV 31 E6 nuclear localization. Although mutation of this putative NLS of HPV31 E6 did not completely block the nuclear localization, it significantly impaired this activity. A tri-partite NLS (NLS1, 2, and 3) has been reported for HPV 16 E6, and mutation of at least two different NLSs was reported to be necessary to completely exclude E6 from the nucleus (Tao et al., 2003). In our studies, mutation of one NLS element at amino acids 121–124 in HPV 31 E6 (corresponding to NLS3 in HPV 16 E6) resulted in a uniform distribution of HPV 31 E6 throughout the cytoplasm and nucleus (Tao et al., 2003). It is possible that additional sequences in HPV 31 E6 besides NLS3 may be required to completely exclude localization to the nucleus, but in our study mutation of this one element was sufficient to impair E6 function. Finally, we cannot exclude the possibility that an uncharacterized factor binds to amino acids 121–124 in HPV 31 E6 and that this activity is also important for E6's role in the viral life cycle. A number of nuclear proteins have been identified as binding partners for E6, and these are involved in a variety of cellular processes such as transcriptional control (c-Myc, ADA3, CBP/p300, IRF-3), DNA synthesis (MCM7), and DNA repair (XRCC-1 and O (6)-MGDMT). It is unlikely that E6TP1 binding is affected by this mutation as its interaction site is located outside the nuclear localization signal. Furthermore, E6TP1 is a cytoplasmic protein. Overall, we believe that binding of E6 to one or more nuclear factors provides an important activity mediated by E6 in the viral life cycle.

Materials and methods

Cell culture

Human foreskin keratinocytes (HFKs) were isolated from neonatal foreskin circumcisions and grown in serum-free keratinocyte growth medium (Clonetics, San Diego, CA) as previously described (Halbert et al., 1992). The untransfected and HPV 31-transfected HFKs were maintained with mitomycin-treated NIH3T3 fibroblast feeders in E-medium (Bedell et al., 1989). E-medium also contains 5% fetal bovine serum and was supplemented with mouse epidermal growth factor (Collaborative Biomedical Products, Bedford, MA) to a final concentration of 5 ng/ml. Prior to harvesting for DNA and protein isolation, NIH3T3 fibroblast feeders were removed with 0.5 mM EDTA in phosphate-buffered saline. SCC-13 cells were maintained in E-medium with fibroblast feeders while 293-T and C33A cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum.

Plasmids

pBR322-HPV31 contains the complete HPV 31 genome sequence inserted into the *Eco*RI site of pBR322. The nucleotide sequence ATA that corresponds to amino acid 128 (isoleucine) was changed to ACC (threonine) through the use of QuickChange™XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described by the manufacturer and confirmed by sequencing. The nucleotide sequence AAA-AAG-AAA-CGA (amino acids 121–124) was changed to GCA-GCG-GCA-GCA,

which encodes four consecutive alanines by the same QuickChange™XL method. The resultant pBR322-HPV31 plasmids containing the desired mutations were then digested with *Spe*I and *Ban*II restriction enzymes to release the 1.2 kb DNA fragment encompassing the entire E6 sequence. These 1.2 kb DNA fragments were then inserted in place of the *Spe*I–*Ban*II fragment of E6 in plasmid containing a complete wild-type HPV 31. This recloning ensured that there were no second site mutations elsewhere in the genomes that could have been generated by the PCR reaction. The 1.2 kb *Spe*I–*Ban*II fragment region of the resultant plasmid was then sequenced to confirm the presence of the desired E6 substitutions. pcDNA3.1(−)31E6 and pcDNA3.1(−)31E6I128T were constructed by inserting PCR fragments containing the wild-type HPV 31 E6 and E6I128T sequences into the *Eco*RI and *Hin*dIII restriction sites of pcDNA3.1(−) vector. pSG5-GFP was constructed by insertion of GFP with PCR generated *Eco*RI restriction sites into the pSG5 vector. Wild-type pSG5-GFP-WTE6 was constructed by inserting E6 with PCR generated *Bam*HI restriction sites into the pSG5-GFP construct. The plasmids pSG5-GFP31E6NLS4A and pSG5-GFP31E6I128T were then generated by using QuickChange™XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described by the manufacturer and confirmed by sequencing. pSG5 and pcDNA3.1(−) vectors were obtained from Stratagene (Stratagene, La Jolla, CA) and Invitrogen (Invitrogen, Carlsbad, CA), respectively. Expression vectors for HA-tagged E6AP (Kao et al., 2000), GST-E6AP (Huibregtse et al., 1993), and myc-tagged E6TP1 (Singh et al., 2003) were described previously.

Generation of stable cell lines

Ten micrograms of pBR322-HPV31 plasmid DNA was digested with *Eco*RI to release the HPV31 DNA sequence. The restriction enzyme was heat-inactivated by incubation at 65 °C for 15 min, and the released viral genome was religated with 800 U of T4 DNA ligase at 15 °C overnight. The religated DNA was then precipitated with isopropyl alcohol and washed with 70% ethanol. The DNA pellet was then resuspended in 10 mM Tris–1 mM EDTA (pH 7.5) and used for transfection. HFKs were grown in 6 cm culture dish to 50–60% confluency in serum-free keratinocyte growth medium (Clonetics, San Diego, CA). One microgram of religated HPV31 genomes was cotransfected with 1 μg of pSV2neo plasmids into HFKs using Fugene (Roche Diagnostics, Mannheim, Germany) transfection reagents as described by the manufacturer. Transfected HFKs were transferred onto mitomycin-treated feeders the following day. Drug selection with G418 (Gibco BRL) started 2 days after transfection according to the following schedules: 200 μg/ml of G418 for 4 days and 100 μg/ml of G418 for 3 days. Drug-resistant colonies were pooled and expanded for analysis.

In-vitro transcription and translation and GST pull-down assay

Rabbit reticulocyte lysate system was used to in-vitro transcribe and translate pSG5-31E6 and pSG5-31E6I128T vectors to produce the HPV 31 E6 and E6I128T proteins radiolabelled with [^{35}S] cysteine (Promega, Madison, WI). The purification of the GST and GST-E6AP fusion proteins was performed as previously described (Lee and Laimins, 2004). Comparable amounts of each [^{35}S] cysteine-labeled E6 protein were incubated with 2 μg of the GST and GST-E6AP fusion protein coupled on glutathione–agarose beads in 250 μl of binding buffer (100 mM NaCl, 100 mM Tris–HCl [pH 8.0], 1% Nonidet P-40 [NP-40], 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride). The mixtures were subjected to shaking at 4 °C for 3 h, and the beads were washed four times with 1 ml of the binding buffer for each wash. The proteins associated with the beads were then released and subjected to SDS–12% polyacrylamide gel electrophoresis. The gels were dried and visualized with autoradiography.

Southern blot analysis

For the transient replication assays, recircularized HPV 31 genomes were transfected into SCC-13 cells and DNA isolated as described previously (Hubert et al., 1999). For the stable replication assay, total genomic DNA was prepared by phenol-chloroform extraction and analyzed by Southern analysis as previously described (Fehrmann et al., 2003).

Transient transfections

For GFP analyses, 293-T cells or C33A cells seeded at approximately 30% confluency in 10 cm dishes were transiently transfected with 6 mg of pSG5-GFP, pSG5-GFP-WTE6, or pSG5-GFP-E6NLS4A using Fugene6 transfection reagents (Roche Diagnostics, Mannheim, Germany). For studies with myc-E6TP1, the myc-tagged E6TP1 expression construct was transfected alone or together with high-risk wild-type and mutant E6 plasmids into C33A cells seeded at approximately 50% confluence in 10 cm dishes using Fugene6 transfection reagents (Roche Diagnostics, Manheim, Germany). The total amount of transfected DNA was maintained constant. After 48 h, cells were harvested and lysates used in Western blot analysis.

Western blot analysis

Whole-cell extracts were prepared in RIPA buffer containing a cocktail of protease inhibitors (Complete, Mini; Roche Diagnostic), and total protein was quantitated with the Bradford assay (Bio-Rad). Equal amounts of protein were electrophoresed on an SDS-polyacrylamide gel, subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) and probed with the following primary antibodies as previously described (Fehrmann et al., 2003): anti-HA (12CA5; Boehringer Mannheim), anti-GFP (JL-8; Clontech, Mountain View, CA), and anti-myc (9E10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins were visualized via enhanced chemiluminescence (Amersham Pharmacia).

Fluorescence microscopy

293-T cells were seeded on BD BioCoat collagen I coated coverslips (BD Biosciences, Bedford, MA) and transiently

transfected using Fugene6 transfection reagent (Roche Diagnostics, Mannheim, Germany) at 30% confluency in 6-well plates. After 24 h, the coverslips were rinsed twice with phosphate buffered saline (Invitrogen, Carlsbad, CA) and fixed for 30 min in 4% paraformaldehyde. Cells were rinsed, permeabilized with 1% Triton-X for 5 min on ice and then rinsed with PBS. Cells were then stained with DAPI for 5 min, rinsed again with PBS and mounted on slides. Images were captured in z-series by a Leica DM IRE2 inverted fluorescence microscope system and processed using Openlab software and deconvolved using Volocity software (Improvision, Lexington, MA).

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